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Legionella petroniana sp. nov., a novel species isolated in Bologna, Italy: taxonomic, genomic and ecological insights in the era of environmental change

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ABSTRACT

This study presents the characterization of a novel *Legionella* species isolated in Italy over three different years from one company and two hospitals. Starting from standard techniques such as culture of water samples, agglutination test, MALDI–TOF MS and gene sequencing analysis used to identify the isolates, genomic and metabarcoding approaches were subsequently employees to further characterize the species. *Legionella* contamination ranged from 400 to 700 CFU/L. The tiny colonies displayed atypical morphology compared to typical *Legionella* features, although they grew on BCYE medium supplemented with L-cysteine. Microscopic and phenotypic analyses revealed Gram-stain negative, Ziehl-Neelsen-negative, rod-shaped, motile cells capable of growing at 32–37 °C, including on selective media such as GVPC and MWY. The isolates tested positive for oxidase and gelatinase activity. Fatty acid profiling identified the dominant components as Summed Features 3 (C16:1 ω7c/C16:1 ω6c, 28.9%), C16:0 iso (18.4%), and C15:0 anteiso (15.4%). Ubiquinone Q13 was the major quinone. Sequence analysis of the *mip* and *rpoB* genes showed 98.2% and 95.1% similarity, respectively, to *L. feeleii* (WO-44C ATCC 35072^T). Whole genome sequencing (WGS) revealed a GC content of 41.5%, a dDDH value of ≤54.9%, and an ANI of 94.06% with *L. feeleii* (WO-44C ATCC 35072^T), supporting the classification of a novel species within the genus *Legionella*. Furthermore, taxonomic resolution of water samples revealed the presence of 168 bacterial genera, including several respiratory, opportunistic, and zoonotic pathogens, as well as seven *Legionella* species. The name *Legionella petroniana* sp. nov. is proposed, with strain 31fl33^T (=DSM 114357^T=CCUG 76442^T) designated as type strain.

Introduction

The surveillance of waterborne pathogens is closely tied to their impact on public health. The recent European Directive on water for human consumption emphasizes the importance of monitoring pathogens with high relevance to human health, introducing the water safety plan (WSP) approach to ensure water quality and reduce infections in communities and healthcare settings.

One of the Directive major innovations is the introduction of *Legionella* among the pathogens that must be mandatory monitored in water for human consumption starting from to high-risk and extending to low-risk buildings, to mitigate its impact on human health.

Despite its importance, *Legionella* remains poorly studied and incompletely characterized, particularly regarding species-level taxonomy, ecological niche, and evolutionary diversity. From a taxonomic perspective, it is possible to distinguish between validated and unvalidated species (Parte et al., 2020).

Legionella spp. are Gram-negative, aerobic bacilli belonging to the unique family *Legionellaceae*, order Legionellales, and genus *Legionella*. These bacteria are free-living and capable of switching between benthic

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and planktonic forms, surviving and replicating within biofilm or protozoa hosts, even under hostile environment condition, such as extreme temperatures, varying pH levels, and exposure to disinfectants (Fields et al., 2002).

In recent years, climate change has been increasingly recognized as a contributing factor impacting the ecology and distribution of *Legionella* spp., particularly by altering environmental conditions such as temperature, rainfall, and water system dynamics. Warmer temperatures in Mediterranean regions have been associated with higher incidence rates of Legionnaires' disease, potentially promoting bacterial survival, replication, and even genetic diversification. Notably, elevated temperatures in drinking water distribution systems can reduce the effectiveness of residual disinfectants, thereby creating favorable conditions for the proliferation of opportunistic pathogens such as *Legionella (*Furst et al., *2024;* Beauté et al., *2016;* Walker, *2018)*.

In both natural and artificial environment, particularly under certain physical, chemical and structural conditions of water distribution system (WDS), *Legionella* can infect humans, leading to two main clinical forms: Legionnaire's disease (LD) and Pontiac Fever, other than a subclinical form (Fields et al., 2002; Centers for Disease Control and Prevention (CDC), n.d.; Rota et al., 2022).

The first reported and most extensively studied species is *Legionella pneumophila* (*Lp*), which includes 15 serogroups (sgs). Currently, 67 out of 74 taxa listed in the List of Prokaryotic Names with Standing in Nomenclature (LPSN) are validly published as *Legionella* species, while the remaining seven taxa have been effectively published but are not yet validly recognized due to the insufficient taxonomic data (as of December 2025) (Parte et al., 2020; Fields et al., 2002; Harrison, and Saunders, N.A.., 1994).

Legionella genus-level classification was historically supported by 16S rRNA and macrophage infectivity potentiator (*mip*) gene sequencing. In recent years, however, whole genome sequencing (WGS) has provided new insights regarding the *Legionella* genus, revealing increased diversity at the species level (i.e., *L. rubrilucens*, *L. erythra* and *L. tauriniensis*, or *L. anisa* and *L. resiliens*) (Fields et al., 2002; Ko et al., 2002; Ratcliff et al., 1998; Birtles et al., 1996; Diogo et al., 1999; Amemura-Maekawa et al., 2004). Genomic analysis has also highlighted the potential pathogenicity of multiple isolates, especially in immunocompromised individuals (Gabrielli et al., 2025).

Although *Lp* sg1 (*Lp*1) accounts for over 90% of cases in Europe and 100% in Italy (Rota et al., 2023; European Centre for Disease Prevention and Control (ECDC), 2023), infections caused by *Legionella* non-*pneumophila* (*L*-np) species remain underestimated. This underreporting is largely due to diagnostic methods for legionellosis, focused almost exclusively on *Lp1*, which reinforces the misperception that it is the unique pathogenic species (Xu et al., 2024; Samson and Maze, 2024; Roussotte and Massy, 2022; Padrnos et al., 2014; Cramp et al., 2010; Vaccaro et al., 2021). As a result, other *Legionella* species, often reported only in environmental samples, are underrepresented in the literature, and their health impacts are poorly understood.

Since all *Legionella* species are capable of intracellular replication in host cells, many of them may be pathogenic under favorable conditions such as immunosuppression, smoking, or advance age (Fields et al., 2002; Herwaldt and Marra, 2018).

To date, 24 of the 67 recognized *Legionella* species are associated with human disease, with most cases involving to *Lp* sg1 (Parte et al., 2020; Bacterio.net, L, 2026; Newton et al., 2010). The most recent ECDC Legionnaires' disease Annual Epidemiological Report (2021) recorded an incidence rate of 2.4 cases per 100,000 population (European Centre for Disease Prevention and Control (ECDC), 2023). Among 1,133 (11%) culture-confirmed cases, 32 (3%) were attributed to *L. anisa*, *L. bozemanii*, *L. longbeachae*, *L. micdadei*, and *L. cincinnatiensis,* while 14 were classified only at the genus level, as *Legionella* species unknown. This persistent underestimation is linked to limited use of culture technique for clinical samples and the lack of diagnostic tools for *L*-np. Furthermore, recent epidemiological and phylogenetic studies have linked cases in northwest Europe to emerging sequence types (STs) of *Lp*, such as ST 1, 23, 37, 47, and 62, originating from different genomic backgrounds (David et al., 2016). Simultaneously, some recent reported cases were associated with new environmental species, suggesting mechanisms of adaptation and spread from natural to man-made environments (Chambers et al., 2021; Muder and Yu, 2002).

Environmental surveillance is essential to identify these ecological niches and to understand the evolutionary mechanisms of *Legionella* species. The number of recognized species and serogroups of Legionellae has significantly increased in the era of next-generation sequencing (NGS), particularly with the widespread use of whole genome sequencing (WGS). However, little is known about the pathogenicity and antibiotic susceptibility of these new species, especially regarding commonly used treatments such as azithromycin and levofloxacin (Dedicoat and Venkatesan, 1999). This is particularly concerning given the absence of standardized antimicrobial susceptibility testing (AST) protocols for *Legionella*, and the fact that most studies still focus exclusively on *Lp*. It is therefore crucial to refine methods for detecting *Legionella*, especially novel species, both in environmental samples and clinical specimens, in order to improve prevention diagnosis, and treatments strategies. Additionally, a deeper investigation into the distribution of *Legionella* in hospital and community settings is needed, considering the selective pressure exerted by climate change and disinfection treatments (Mazzotta et al., 2021). WGS plays an essential role in identifying sources of infection, transmission routes, and the emergence of the new clones and species with pathogenic potential.

This study presents the taxonomic and genotypic characterization, of strains isolated in different years from different WDSs in one company and two hospitals (strains 31fI33^T, 29fVS95, and 28fT52) during a routine surveillance program. These facilities, in compliance with national *Legionella* guidelines and workers safety directive (Dlgs 81/2008), have implemented a *Legionella* risk assessment plan since 2015 and, in accordance with the new directive on water for human consumption developed a new water safety plan (Italian Work Ministry Legislative Decree 09.04.2008, 2008; Italian Republic, 2023; European Parliament the Council of the European Union, 2020).

In this context, the study proposed an integrated self-assessment surveillance approach that goes beyond standard culture and identification methods, combining phenotypical and genomic characterization of isolates with the description of their water samples microbiomes.

This comprehensive methodology led to discovery of a novel species within the genus *Legionella,* proposed as *Legionella petroniana* sp. nov., with strain 31fI33^T (= DSM 114357^T = CCUG 76442^T) designed as type strain.

Materials and methods

The taxonomic workflow presented describes the routine laboratory practices, starting from isolation, culture and phenotypic and proteomic characterization of the isolates. The preliminary data obtained were than associated with gene sequencing analysis to assess the isolates identification, and finally WGS and comparative genomic analyses were performed to formally resolve the taxonomic status.

Water distribution systems (WDSs) description, Legionella isolation and evaluation of growth conditions

Several *Legionella*-like isolates were detected during a self-assessment *Legionella* environmental surveillance, conducted in a company and two hospitals located around Bologna city, Italy, from 2015 to 2024 according to the Italian and regional guidelines for *Legionella* prevention and control (Emilia-Romagna Region Regional Guidelines for Surveillance and Control of Legionellosis, 2012; *Italian National Institute of Health Guidelines for Prevention and Control of Legionellosis,* 2015). Starting from a first observation occurred in 2018 in a company, other isolates sharing the same morphological and phenotypical

characteristics, that will be above discussed, were subsequently found.

The main characteristics of WDSs, where the isolates were detected, and the sampling plan adopted by facilities are described in Table 1. For privacy reasons, the facilities involved in the study are labelled as A= company, B= Hospital 1, C= Hospital 2. The hot and cold-water samples (1 Liter) were collected from outlets distributed along the WDS, following the UNI EN ISO 19458:2006 in post-flushing modality (UNI EN ISO 19458, 2006). During sampling, temperature and disinfectant residue levels (if present) were measured at each outlet and recorded. In detail the temperature (°C) of samples was measured using a digital thermometer for probes (XS Temp 7 Vio PT 100 Thermometer from –200 to +999 °C; Eutech Instruments Pte Ltd., Singapore) the and residues of disinfectant (H_2O_2/Ag^+) was measured (mg/L) on-site by a commercial kit that reveals the residual H_2O_2 component used a colorimetric test. The *Legionella* isolation was performed using standard culture technique in accordance with ISO 11731:2017, using the washing membrane procedure (UNI EN ISO 11731, 2017). The media used for isolation was Glycine, Vancomycin, Polymyxin B, Cycloheximide (GVPC) agar medium (Thermo Fisher Scientific, Diagnostic, Ltd., Basingstoke, UK), while the sub-cultures of the isolates were performed on Buffered Charcoal Yeast Extract (BCYE) agar with L-cysteine (Cys+) and without L-cysteine (Cys-), respectively. The plates were incubated at 35.0 ± 2 °C with 2.5% CO_2, and the *Legionella* growth was assessed every two days, until 15 days. Contamination levels were expressed as colony-forming units/liter (CFU/L), and the data were reported as mean concentration ± SE.

The putative *Legionella* colonies, based on ability to grow only on BCYE Cys+ were then sub-cultured and stored at -80 ± 2 °C in glycerol, for further analysis (UNI EN ISO 11731, 2017).

Different temperatures and media growth conditions were evaluated. Specifically, the following temperatures were tested: 32, 35.0 and 37 °C, with and without 2.5% of CO_2, as well as under microaerophilia conditions.

The growth of isolates was assessed on GVPC, Wadowsky Yee Medium (MWY), tryptone soya agar (TSA) with 5% sheep blood agar medium (Thermo Fisher Scientific, Diagnostic, Ltd., Basingstoke, UK) and Chocolate Enriched Agar Medium (MEUS S.r.L., Piove di Sacco, Padova, Italy) to observe their growth rate and morphology.

The morphology of the colonies was studied by Heerbrugg Wild M38 Professional Optical Stereo Binocular Microscope with Volpi Intralux 4000 Light Source (90W).

The presence of *P. aeruginosa* contamination was evaluated in the same samples, considering its role on biofilm component that could interfere with *Legionella* isolation during the culture-based techniques. *P. aeruginosa* analysis was performed according to the UNI EN ISO 16266:2008 standard using the membrane filtration technique, with Pseudomonas C-N Selective Agar (Thermo Fisher Scientific, Diagnostics, Ltd., Basingstoke, UK) (UNI EN ISO 16266, 2008).

Table 1
Summary of distribution water systems' characteristics of each facility.

Facilities	A	B	C
Origin	Company	Hospital 1	Hospital 2
Year of strain isolation	2018	2019	2021
Type of water supplied	Municipal	Municipal	Municipal
Softener treatment	Yes	Yes	Yes
Disinfection treatment applied	None	UV Lamp	Yes (H_2O_2/Ag^+)
Hot water characteristics	Two hot water tanks, in parallel connected (500 liters each boiler)	One hot water tank (905 liters)	One hot water tank (3000 liters)
Surveillance program	Annual	Semestral	Semestral

Identification of Legionella isolates by Agglutination test and MALDI–TOF MS

The *Legionella* colonies, grown exclusively on BCYE Cys+ plates, were identified using the *Legionella* latex agglutination test kit (Thermo Fisher Scientific, Ltd. Basingstoke, UK), following the manufacturer's instructions. This test discriminates among *Lp*1, *Lp* serogroups 2–14 (*Lp* 2-14) and seven *L*-n*p*.

The identification was obtained through the Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Biotyper® system (Bruker Daltonik GmbH, Bremen, Germany), as previously described (Pascale et al., 2020; Girolamini et al., 2025). Briefly, a biomass from each biological replicate of colonies was harvested and spotted in triplicate on a target plate. The colonies were identified using the three sample preparation methods (Girolamini et al., 2025). Spectra acquisition and processing were conducted with the Microflex LT mass spectrometer (operating in linear positive mode, 2,000–20,000 Da) and the MALDI Biotyper Compass software (v.4.1.1), utilizing a library (version BDAL 7854) containing spectra from 48 *Legionella* species main spectra, derived from both reference and environmental strains. Data interpretation followed the manufacturer's guidelines: species-level identification (log score ≥ 2.00, high-confidence score), genus-level identification (log score between 1.70 and 1.99, low-confidence score), and not identified (log score between 0.00 and 1.69). Additionally, dendrograms based on Hierarchical Cluster Analysis (HCA) of the MALDI Biotyper® spectra were generated using MALDI Biotyper Compass Explorer software (v.4.1.1). These dendrograms employed 31fl33^T spectra and the isolates included in the MALDI Biotyper® spectra database to build a tree-like structure linking the *Legionella* strains via a linkage algorithm.

Physiology and chemotaxonomy

Three colonies, randomly selected from multiple colonies grown in each positive sample were selected for subsequent analysis, indicated as follows: 31fl33^T representative of *Legionella* reported in the company, 29fVS95 and 28fT52 for Hospital 1 and 2, respectively. Moreover, *L. feeleii* ATCC 35072^T (the most related strain), and *Legionella pneumophila* sg1 strain Philadelphia-1 ATCC 33152^T (*Lp*1 ATCC 33152^T, the most virulent strain), were used for comparative analysis. All of them were sub-cultured on BCYE Cys+ agar at 35.0 ± 2 °C with 2.5% CO_2. The main features of the most closely related strains within the identified clade were also obtained from reference literature.

Gram staining and Ziehl-Neelsen staining were performed on the isolates, and the presence or absence of autofluorescence was evaluated under a Wood's lamp (long-wavelength UV light at 365 nm).

Additionally, the following biochemical characteristics were assessed: oxidase activity using oxidase test strips (Biolife, Milan, Italy) and catalase activity using the catalase Colorimetric Activity Kit (Thermo Fisher Scientific Diagnostic, Ltd., Basingstoke, UK). The Diatabs kit and Nutrient Gelatin medium (Biolife, Milan, Italy) were used to test hippurate hydrolysis and gelatinase activity, respectively. Further biochemical profiling was conducted using the BBL™ Crystal™ Enteric/Non-Fermenter ID kit (Becton Dickinson Systems, Cockeysville, MD, USA) and the Remel™ RapID™ NF Plus system (Thermo Fisher Diagnostic) following the manufacturer's instructions. β-lactamase production was detected using the Oxoid™ Nitrocefin Solution (Thermo Fisher Scientific Diagnostic, Ltd., Basingstoke, UK).

In addition, analysis of the composition of cell wall fatty acids (CFAs), isoprenoid quinones, polyamines and lipids of strains 31fl33^T, 29fVS95, 28fT52, and *L. feeleii* strain 691-WI-H DSM 25316^T (*L. feeleii* DSM 25316^T) was analyzed by Identification Services of Leibniz-Institut DSMZ – Deutsche Sammulung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The polyamine profile was obtained by extracting 50-60 mg of wet biomass and analyzing it via gas

chromatography–mass spectrometry (GC-MS). The polyamines and their precursors screened include: agmatine, cadaverine, homospermidine, norspermidine, 1,2- and 1,3-diaminopropane, putrescine, N-acetyl-putrescine, spermidine and spermine. Data for the most closely related *Legionella* strains and *Lp*1 subs. *pneumophila* Philadelphia 1 (CCUG 9568^T), used as a positive control, were obtained from the literature (Crespi et al., 2023; Lambert and Moss, 1989; Thacker et al., 1989; Adenike et al., 2001; Dennis et al., 1993). No literature data are available regarding the fatty acid composition of *L. massiliensis* and *L. tunisiensis*, as well as of the ubiquinone content of *L. fairfieldensis* and *L. massiliensis*, therefore these data are missed.

DNA extraction and gene sequencing

The 31fI33^T, 29fVS95 and 28fT52 isolates were processed for gene sequencing of the *mip*, RNA polymerase beta subunit (*rpoB*), and 16S rRNA genes (Ko et al., 2002; Ratcliff et al., 1998; Yong et al., 2010).

DNA extraction was performed using the InstaGene Matrix (Bio-Rad, Hercules, CA, USA) and it was quantified with a Qubit fluorometer (Thermo Fisher Scientific, Paisley, UK). Currently, the *mip* gene is the gold standard for the identification of *Legionella spp*., for both clinical and environmental samples (*Italian National Institute of Health Guidelines for Prevention and Control of Legionellosis*, 2015). It encodes for a 24 kDa surface protein that acts as a key virulence factor in the invasion of host cells by *Legionella* (Cianciotto et al., 2006). The *mip* gene sequencing protocol followed by the guidelines provided by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for *Legionella* Infections (ESGLI) (Ratcliff et al., 1998; Fry et al., 2007).

Additionally, the *rpoB* gene sequencing was used on isolates to improve the identification level among the *Legionella species*, as previously demonstrated (Pascale et al., 2021). This gene encodes a subunit of DNA-dependent RNA polymerase, which contains a highly conserved region across bacteria and can be used for an accurate bacterial classification (Kim et al., 1999). The PCR amplification protocols for the *mip* and *rpoB* genes were performed as previously described (Ko et al., 2002; Ratcliff et al., 1998; Girolamini et al., 2022a).

Although the use of the 16S rRNA gene for *Legionella* identification has been largely surpassed due to the low discrimination power in interspecies evaluation, it was included in this study following the protocol described by Rafiee et al. given its relevance for the description of novel species (Rafiee et al., 2014). After purification using ExoSAP-ITTM PCR Product Cleanup kit (Applied Biosystems, Foster City, CA), the *mip*, *rpoB* and 16S rRNA amplicons were sequenced using BigDye™ Chemistry and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Raw sequencing data were assembled using CLC Main Workbench 22.0.2 software (QIAgen, Hilden, Germany). To assess the genetic similarity between strains isolated and other *Legionella* species, official recognized (Parte et al., 2020; Schoch et al., 2020) and available in culture collections, Basic Local Alignment Search Tool (BLAST) research were carried out through the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The PCR amplicon of *mip* (611 bp), *rpoB* (329 bp) and 16S rRNA (1468 bp) genes were used to identify the best matches among *Legionella* species. ESGLI has established an accessible web database (http://bioinformatics.phe.org.uk/cgi-bin/Legionella/mip/mip_id.cgi) containing sequence data from validated *Legionella* species and allows the identification of *Legionella* species. Accordingly, the obtained *mip* sequences were compared to those in the *Legionella mip* gene sequence database using a similarity analysis tool. However, the database is currently under development and is not available for external access, although it remains accessible internally to database curators at UKHSA (legionella-sbt@ukhsa.gov.uk). Species-level identification based on the *mip* gene sequence was conducted according to the classification scheme proposed by Ratcliff et al., applying a similarity threshold of >98.0% (Ratcliff et al., 1998; Fry et al., 2007). The *rpoB* gene sequences were compared with type strain sequences deposited in the NCBI database from various culture collections, including the American Type Culture Collection (ATCC), National Collection of Type Cultures, Central Public Health Laboratory (NCTC), NITE Biological Research Center, National Institute of Technology and Evaluation (NBRC), and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), among others. For species-level identification, the new *Legionella* classification scheme targeting the *rpoB* gene, developed by Pascale et al. and based on a 329 bp gene fragment, was applied using a similarity threshold of >95.2% (Pascale et al., 2021).

For the 16S rRNA gene, species-level identification was performed based on sequence similarity, using a threshold set at >97% (Stackebrandt and Goebel, 1994). However, a more recent study by Stackebrandt & Ebers proposed a higher cut-off value of >98.7% for more accurate species discrimination (Stackebrandt and Ebers, 2006). In addition, complete gene sequences obtained through WGS results were used to further assess gene sequence similarity.

Phylogenetic analyses based on gene sequences

To assess the phylogenetic relationship among strains 31fI33^T, 29fVS95 and 28fT52 and the other *Legionella* species, multiple sequence alignment (MSA) was performed, followed by the construction of phylogenetic tree based on *mip*, *rpoB* and 16S rRNA genes sequences. Considering the high number of validated *Legionella* species (n=67), for graphical reasons the phylogenetic trees were built using only *Legionella* species belonging to the clade most closely related to 31fI33^T, 29fVS95, and 28fT52 strains. The trees were rooted using the closely related genus Coxiella, represented by *Coxiella burnetii* species, strain RSA 493, as an outgroup.

When necessary, sequences were trimmed to match the length of the reference. In addition, BLAST searches were conducted on the NCBI database, and the top ten matching strain sequences were retrieved for comparison. Multiple sequence alignments were carried out using the MUSCLE algorithm (Edgar, 2004), implemented in Geneious Prime (v2023.0.4; http://www.geneious.com) (Kearse et al., 2012), using default parameters. Phylogenetic trees were constructed from the aligned sequences using the Bayesian Evolutionary Analysis by Sampling Trees (BEAST, v1.10.4) software (Drummond and Rambaut, 2007). The bayesian Evolutionary Analysis Utility (BEAUti) (v1.10.4) was used to select the consensus trees (Drummond et al., 2012).

Whole genome sequencing and Genome Features

WGS of 31fI33^T, 29fVS95 and 28fT52 strains were performed as previously described (Girolamini et al., 2022a; Girolamini et al., 2022b). NGS libraries were prepared using 100 ng of genomic DNA with the Nextera XT DNA library prep kit (Illumina, New England Biolabs, Ipswich, MA, USA). Sequencing was conducted on the Illumina NextSeq 500 platform using 2x250 bp paired-end reads. Sequencing reads from strain 31fI33^T were processed using the TORMES pipeline (v.1.2.0) with default settings (Quijada et al., 2019), resulting in a draft genome assembly. The pipeline included quality filtering with PRINSEQ (v0.20.4) and de novo assembly using SPAdes (v13.4.1) (Bankevich et al., 2012). The resulting draft genome was scaffolded using CSAR (v1.1.1) (Chen et al., 2018), applying a reference-based approach with *L. feeleii* NCTC 11978^T (GCF_900461565.1) as the reference genome. Further refinements were performed using Geneious Prime (v2022.0.2; http://www.geneious.com) by remapping reads to the CSAR-generated scaffolds. After quality assessment using FastQC (v. 0.11.9), raw reads from strains 29fVS95 and 28fT52 were trimmed using Trimmomatic (v0.39) in paired-end mode using default parameters. Trimmed reads were assembled de novo using SPAdes (v3.13.0). Assembly quality was evaluated using QUAST (v5.2.0), which was used to calculate standard assembly statistics, including the number of contigs, total genome length, GC content, and N50/L50 values. Genome completeness for all three strains was evaluated using Benchmarking Universal Single-Copy

Orthologs (BUSCO (v5.0.0) in prokaryotic genome mode with the legionellales_odb10 lineage dataset, and gene prediction was performed using Prodigal (Seppey et al., 2019). Final draft genomes were submitted to GenBank and annotated using the PGAP pipeline (v4.3) (Tatusova et al., 2016). Genome completeness and contamination were further assessed using CheckM2 (v1.1.0) (Chklovski et al., 2023).

Genome-based taxonomy and phylogenomics

Genome-based analyses were conducted to support genus assignment, species delineation and phylogenomic placement.

The percentage of conserved proteins (POCP) was calculated as a matrix across strains 31fI33^T, 29fVS95 and 28fT52 and 65 validated *Legionella* reference genomes annotated in NCBI, using the POCP-matrix Python tool (v2.3.6). This analysis supports genus-level placement and compare conserved proteome fractions among closely related taxa (Qin et al., 2014). To assess overall genome similarity, an amino acid-based comparative analysis was performed using average amino acid identity (AAI), calculated with EzAAI tool (v1.2.1 http://leb.snu.ac.kr/ezaai/download) (Schober et al., 2025; Kim et al., 2021), comparing the isolates with 65 *Legionella* species genomes. The resulting data were visualized in a heatmap generated using Python script (v3.10.4) using Matplotlib (v3.6.3) (Thomas et al., 2023), Pandas (v1.4.2) (Reback et al., 2022) and Seaborn (Waskom, 2021) (v0.12.2) libraries. AAI and POCP were used as complementary genus-level relatedness metrics, and their interpretation was supported by genome-based phylogenies rather than by the application of fixed universal thresholds (Qin et al., 2014; Konstantinidis and Tiedje, 2005).

Although guidance thresholds have been proposed for genus delineation using AAI (e.g., >60–65%) and POCP (e.g., >50%), multiple studies have shown that genus-specific boundaries can widely vary and may shift across different taxa, including within the same family (Riesco and Trujillo, 2024). Given the widespread distribution and deviation of POCP values, the use of a general genus threshold is not recommended; instead, family-level patterns can be more informative, provided that a comprehensive genome-based phylogeny supports the taxonomic conclusions (Riesco and Trujillo, 2024). In this context, POCP is not discouraged, as it generally correlates with AAI, but it is best used as a complementary measure because it shows higher variability and can be influenced by marked differences in genome sizes; therefore, POCP comparisons should preferentially be performed among genomes of broadly similar sizes (Riesco and Trujillo, 2024).

Genomic similarity between the draft genomes was evaluated using the OrthoANI tool (Yoon and Ha, 2017). Average nucleotide identity (ANI) was also calculated using FastANI (Jain et al., 2018) via the DFAST platform (Tanizawa et al., 2018), comparing each genome against 13,000 prokaryotic reference genomes from NCBI. Additional comparison included ANIb and ANIm, calculated using JSpeciesWS (v3.9.8; https://jspecies.ribohost.com/jspeciesws/#home) (Richter et al., 2016), employing BLAST+ and MUMmer as alignment algorithms. Phylogenetic relationships were further analyzed using digital DNA-DNA hybridization (dDDH) via the Genome-to-Genome Distance Calculator (GGDC, v3.0; https://ggdc.dsmz.de/ggdc_background.php, accessed on 20 February 2025) (Meier-Kolthoff et al., 2022), default parameters were used. The dDDH estimate was based on formula 2, which is recommended for incomplete draft genomes and is independent of genome length. BLAST+ was used as the local alignment tool (Camacho et al., 2009). Comparative analyses include our strains and *L. feeleii* (ATCC 35072^T), identified as the closest relative based on ANI results.

Genome-based classification and phylogenomic inference were additionally performed using the Type (Strain) Genome Server (TYGS) platform, which implements the Genome BLAST Distance Phylogeny (GBDP) approach. Genome placement within the Genome Taxonomy Database (GTDB) framework was evaluated using GTDB-based phylogenomic analysis.

Phylogenetic placement of the new bacterial species was performed using the Genome Taxonomy Database Toolkit (GTDB-Tk) v2.3.2 (Allen et al., 2017). The genome sequence of the proposed new species was analyzed alongside the reference genomes in the GTDB using a set of conserved single-copy marker genes.

Phylogenetic relationships with 65 other *Legionella* species annotated in NCBI were investigated using the Codon Tree pipeline in the Bacterial and Viral Bioinformatics Resource Center (BV-BRC, v3.30.19; https://www.bv-brc.org/app/PhylogeneticTree) (Olson et al., 2023). Protein sequences were aligned using MUSCLE (Edgar, 2004), while coding nucleotide sequences were aligned using the Codon Align function of BioPython (Cock et al., 2009). A concatenated alignment of all protein and nucleotide sequences was generated with MAFFT and analyzed using RaxML (v8.2.11) (Stamatakis, 2014; Stamatakis et al., 2008). The WGS-based phylogenetic tree was rooted using the closely related genus *Coxiella*, represented by *Coxiella burnetii* RSA 493, as an outgroup. The tree was visualized using FigTree (v1.4.4) (Rambaut, 2007). The genome sequences used for this analysis are listed in Table S1 in the Supplementary Information.

Fingerprinting and clonality study

The clonality of the isolates was evaluated using RAPD-PCR, including both REP-PCR and $(GTG)_5$-PCR fingerprinting. For amplification, 100 ng of genomic DNA was used as a template with the REP1R-Dt (3'-CGGNCTACNGCNGCNIII-5') and REP2-Dt (3'-CATCCGGNC-TATTCNGCN-5') primers, following the protocol described by Georghiou et al., as well as the $(GTG)_5$ primer (5'-GTGGTGGTGGTGGTG-3'), according to Colautti et al. (Colautti et al., 2024; Georghiou et al., 1994). The optimal annealing temperature for (GTG)s-PCR was set at 44 °C. Amplicons were separated by electrophoresis on a 2% (w/v) agarose gel. The *L. resiliens* 8cVS16^T (DSM 114356^T) strain was used as a positive control. In addition, single nucleotide polymorphisms (SNPs) differences among the three strains were determined using Snippy (v4.6.0; https://github.com/tseemann/snippy).

Core genome analysis

The core genome analysis included comparisons with the most closely related *Legionella* species and the highly virulent *Lp*1 reference strains. Genomic data for these reference strains were retrieved from NCBI and compared with the genomes of strains 31fI33^T, 29fVS95 and 28fT52. To further investigate genome similarities, the BLAST Ring Image Generator (BRIG) (v. 0.95) (Alikhan et al., 2011) was used to compare the genome of 31fI33 ^{T}, 29fVS95 and 28fT52 strains against *L. feeleii* (ATCC 35072^T), and *Legionella pneumophila subs. pneumophila Philadelphia serogroup 1* (*Lp*1, ATCC 33152^T), using 31fI33 ^{T} as the reference. This allowed an in-depth qualitative visualization of genomic differences, showing regions of complete identity with the reference genome using different color patterns. Moreover, using an in-house Python script was calculated the percentage of coverage.

Genome size and sequence variations were further analyzed using the Prokka annotation pipeline (v1.14.6), and missing genes among the genomes were identified using a custom in-house Python script. Pan-genome analysis was performed using Roary software (v3.13.0) (Sitto and Battistuzzi, 2020) to identify shared and accessory genes among closely related *Legionella* species.

Genes were classified as follows:

- Total genes (present in 0% to 100% of the strains);
- Core genes (present in 99% to 100% of the strains);
- Soft core genes (present in 95% to <99% of the strains);
- Shell genes (present in 15% to < 95% of the strains);
- Cloud genes (present in 0% to < 15% of the strains).

Virulence, pathogenicity and antibiotics resistance genes analysis

Genome annotation was initially performed using the Rapid Annotation using Subsystem Technology (RAST, v2.0) server, which provided a broad functional classification of genes, including the preliminary identification of potential virulence-associated genes (Fang et al., 2021). For more targeted and comprehensive assessment, putative virulence factors were subsequently screened against the Virulence Factors Database (VFDB), and only hits validated by VFDB, were considered for detailed virulence profiling (Liu et al., 2022). Antibiotic resistance genes were identified using ABRicate, which screened the genome against three curated databases: ARG-ANNOT (v28 July 2019) (Gupta et al., 2014), CARD (v2.1.2) (Alcock et al., 2019) and ResFinder (v3.2.0) (Zankari et al., 2012). Finally, the potential pathogenicity of isolates was predicted using PathogenFinder (v1.1) (Cosentino et al., 2013). Unless otherwise specified, all bioinformatic tools were executed using default parameters.

16S rRNA gene (V3-V4) metabarcoding analysis of water samples

Sample collection and DNA preparation

Additional one-liter water samples were collected from the same sampling points used for culture-based analyses, corresponding to the location-samples where strains 31fI33^T, 29fVS95 and 28fT52 were isolated, as well as from random sampling points within the facilities. Genomic DNA was extracted by filtering 1 L of water through a 0.22 µm polyethersulfone (PESU) membrane, followed by processing with the DNeasy PowerWater Extraction Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. The extracted DNA was stored at -20°C for subsequent sequencing analyses.

V3–V4 16S rRNA gene metabarcoding: amplification, library preparation, and sequencing workflow

DNA quantification was performed using the Qubit™ 4 Fluorometer and the dsDNA Broad Range Quantitation kit (Fisher Scientific Italia, Segrate, Italy), following the Illumina 16S Metagenomic Sequencing Library Preparation Guide (https://emea.support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf, accessed on February 8, 2024). For bacterial taxonomic profiling, the V3–V4 region of the 16S rRNA gene was amplified using the primer pair S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013). Indexed libraries were generated via limited-cycle PCR using Nextera technology (Illumina, San Diego, CA, USA). Libraries were subsequently purified with VAHTS DNA Clean Beads (Vazyme, Red Maple Hi-tech Industry Park, Nanjing, PRC), pooled at equimolar concentrations (4 nM), denatured, and diluted to a final concentration of 5 pM. Sequencing was performed on the Illumina MiSeq platform using the MiSeq Reagent Kit V3 (Illumina, San Diego, CA, USA) with a 2 × 300 bp paired-end protocol, according to the manufacturer's instructions. The expected amplicon size for the V3–V4 region was approximately 428 bp, suitable for paired-end sequencing on the MiSeq system.

Data Processing and 16S rRNA gene metabarcoding community profiling

Microbiota samples were processed using QIIME2 (version 2020.6) (Bolyen et al., 2019). The Divisive Amplicon Denoising Algorithm 2 (DADA2) plugin was used to remove noise and chimeras, generating Amplicon Sequence Variants (ASVs) (Hall and Beiko, 2018). Quality filtering and sequence clustering were performed with VSEARCH (v2020.6.0) (Rognes et al., 2016). Taxonomic classification of high-quality reads was conducted using the SILVA reference database (v132) (Yilmaz et al., 2014). The resulting data were imported into R (v4.2.2) (R Core Team R, 2022) and analyzed in RStudio (v2022.07.2 Build 576) (RStudio Team RStudio, 2020). Downstream analyses were carried out using various R packages, including *phyloseq* (Callahan et al., 2016; McMurdie et al., 2013), *rbiom* (Smith, n.d.), *ggplot2* (Wickham, 2016), *tidyverse* (Paradis et al., 2004; Wickham et al., 2019), *tidyr* (Wickham et al., 2014), *ggpubr* (Kassambara, n.d.), and *dplyr* (Wickham et al., 2023). All available high-quality reads were included in the analyses without rarefaction, to estimate the relative abundance of taxa within each sample. A stacked bar plot was generated to visualize the relative abundance of taxa at the genus level, displaying only genera with an average read count greater than five for clarity. This amplicon-based 16S rRNA gene metabarcoding approach was used to characterize the microbial community composition associated with the sampling sites, rather than to reconstruct metagenome-assembled genomes (MAGs). In addition, the Protologger web tool (accessible at https://www.protologger.de/) was used to analyze the presence of our genomes in MAG studies across diverse environments (Hitch et al., 2021).

Using the 16S rRNA gene sequences, the genomes were matched against a database containing approximately 19,000 amplicons and then compared to 49,094 high-quality MAGs derived from publicly available metagenomic datasets. Although this approach does not allow strain-level resolution within the genus *Legionella*, it provides a framework for exploring potential ecological associations at higher taxonomic levels. The tool permits the screening of strain-specific genomes against metagenomic datasets, allowing for the investigation of potential ecological niches and environmental distribution of microbial strains. Moreover, Protologger provides information on the functional repertoire of input genomes, including carbohydrate-active enzymes (CAZy), thereby enabling the characterization of enzymatic systems involved in carbohydrate metabolism and modification.

Antibiotic susceptibility test

Antibiotic susceptibility pattern of strain 31fI33^T was assessed using both the MIC Test Strip and Broth Microdilution (BMD) methods, despite the absence of defined epidemiological cut-off (ECOFF) values for *Legionella* (Sewell et al., 2025). The MIC Test Strip (Liofilchem, s.r.l, Roseto degli Abbruzzi (TE), Italy) assay was performed on subcultures grown on BCYE Cys + agar at 35 °C in a humidified atmosphere with 2.5% of CO_2 for 48h. The bacterial biomass was resuspended in sterile water to achieve a 0.5 McFarland turbidity standard. The suspension was evenly spread onto BCYE Cys+ agar plates using a sterile swab, and a single gradient strip was applied to the medium. This procedure was repeated for each of the following antibiotics: azithromycin (0.016-256 mg/L), erythromycin (0.002-32 mg/L), ciprofloxacin (0.016-256 mg/L), rifampicin (0.016-256 mg/L), tigecycline (0.002-32 mg/L) and imipenem (0.002-32 mg/L). The tested concentration ranges are indicated in parenthesis. Plates were incubated at 35 °C for 48h in a humidified atmosphere. MICs were determined by identifying the point at which the ellipse of inhibition intersected the antibiotic gradient strip. Interpretation of MIC values was based on EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidance, as provided by ESCMID (EUCAST, 2021). The BMD assay was carried out using a bacterial suspension prepared in Liquid Growth Medium (LGM) without charcoal to prevent antibiotics inactivation (Sewell et al., 2025; Portal et al., 2021), adjusted to a 0.5 McFarland turbidity standard. In 96-well microtiter plates, 40 µL of different antibiotics concentrations were mixed with 160 µL of bacterial suspension. The final bacterial concentration was approximately 4×10^5 to 5×10^5 cells/mL. Each row of the plate contained a different antibiotic at the follow concentration range: azithromycin (0.0075-16 mg/L), erythromycin (0.0075-16 mg/L), ciprofloxacin (0.00025-0.512 mg/L). Plates were incubated at 35°C for 48 h in a humidified atmosphere. MICs values were interpreted by comparing the results with MIC distribution table provided in the EUCAST guidelines (EUCAST, 2021).

Results and discussion

Over the years, during multiple sampling sessions conducted in three

different facilities (Facility A, B, and C) in the Bologna area, colonies displaying morphological features distinct from the typical one *Legionella* spp. were observed. Among the sampling sessions in which these atypical colonies were identified, three sampling dates (October 2018, July 2019 and April 2021) and three representative isolates were selected for further characterization: strain 31fI33^T from Facility A, strain 29fVS95 from Facility B, and strain 28fT52 from Facility C. The outlets (sampling points) from which the isolates were recovered, together with the isolate IDs, the level of *Legionella* and *P. aeruginosa* contamination detected in the facilities, and the values of the main physical and chemical parameters measured during sampling, are reported in Table 2. In Facility A, *Legionella* was detected only in the cold WDS (280.00±195.96 CFU/L), while *P. aeruginosa* was detected only in the hot WDS (8.70±5.59 CFU/100mL). In Facility B, *Legionella* or *P. aeruginosa* were absent from the cold WDS. In the hot WDS, *Legionella* reached 160±136.38 CFU/L, and *P. aeruginosa* was present at 20±20 CFU/100mL. Facility C was the only one with disinfection treatment in the hot WDS (H_2O_2/Ag^+, 10-25 mg/L). Despite this, *Legionella* reached the highest level in the hot WDS (1125.00±666.72 CFU/L), while *P. aeruginosa* was not detected. Strain 31fI33^T was isolated from a cold-water outlet, located on the terrace, used for the air handling unit (AHU) maintenance (19.1 °C, with *Legionella* contamination of 400 CFU/L) potentially posing a high risk of *Legionella* aerosolization. Strain 29fVS95 was isolated from a shower in the women's changing room (47.8 °C, with *Legionella* contamination of 700 CFU/L), and strain 28fT52 from a restroom sink (48.0°C, with *Legionella* concentration of 400 CFU/L, of 10-25mg/L disinfectant concentration). *P. aeruginosa* was absent in all isolates.

Table 2
Hot and cold-water *Legionella* and *P. aeruginosa* contamination, temperature and disinfectant residues, measured in facilities A, B and C. Data are presented as mean±SE.

Facility	A	B	C
Sampling date	October 2018	July 2019	April 2021
	Hot water system		
Temperature (°C)	41.77±0.98	47.24±1.97	46.37 ±1.50
Legionella concentration (CFU/L)	Absent (<100)	160±136.38	1125.00 ±666.72
P. aeruginosa concentration (CFU/100mL)	8.70±5.59	20±20	Absent (<1)
Disinfection treatment applied (mg/L)	Absent	Absent	H_2O_2/Ag^+ 10-25
	Cold water system		
Temperature (°C)	18.70±0.25	24.7	20.36 ±1.73
Legionella concentration (CFU/L)	280.00±195.96	Absent (<100)	Absent (<100)
P. aeruginosa concentration (CFU/100mL)	Absent (<1)	Absent (<1)	Absent (<1)
Isolate name	31fI33^T	29fVS95	28fT52
Sampling point location	single cold-water outlet on the building's terrace, used for cleaning and maintenance of the air handling unit (AHU)	shower in the women's changing room	sink in a restroom
Temperature (°C)	19.1	47.8	48.0
Legionella concentration (CFU/L)	400	700	400
Disinfectant concentration (mg/L)	/	/	10-25
P. aeruginosa concentration (CFU/100ml)	Absent (<1)	Absent (<1)	Absent (<1)

Isolation of bacterial strains, growth condition and identification

The *Legionella*-like colonies grew exclusively on BCYE C*ys*+, GVPC, and MWY, with no growth observed on any other tested media (Fig. S1). No significant differences in growth were observed across an incubation temperature range of 32-37 °C. The colonies showed rapid growth, appearing as early as 24 hours post-incubation, with no notable variation between aerobic, CO_2-enriched, or microaerophilic condition. Morphologically, the colonies were convex, light taupe in color, round, and approximately 1mm in diameter (Fig. 1).

The strains did not exhibit autofluorescence under Wood's lamp (long-wavelength UV light at 365 nm) and returned negative results using a commercial *Legionella* agglutination test. Initial identification via the MALDI Biotyper® System, using the manufacturer's standard library, classified the strains as *L. feeleii*, albeit with a low confidence score: 1.90 for 31fI33^T, 1.76 for 29fVS95, and no identification for 28fT52. Upon adding strain 31fI33^T to the in-house library and re-analyzing all three isolates, the identification score increased significantly to ≥2.00 Specifically, the revised scores were 2.57 for 31fI33^T, 2.50 for 28fT52, and 2.00 for 29fVS95, indicating reliable species-level identification (Girolamini et al., 2025). The HCA dendrograms generated from MALDI-TOF MS spectra (Figs. 2 and S2) revealed the relationships among strains 31fI33^T, 29fVS95, and 28fT52, based on ribosomial protein profiles, as well as their positioning relative to other *Legionella* species included in the instrument database. Strains 31fI33^T and 28fT52 closely clustered, while 29fVS95 was slightly more distantly related. The dendrogram also identified a monophyletic group composed of *L. feeleii, L. jordanis*, *L. fairfieldensis*, and *L. longbeachae*, with the three studied isolates regrouped in a distinct clade. These findings suggest that the isolates 31fI33^T, 28fT52 and 29fVS95 are evolutionary related to this group, they are genetically distinct, potentially representing a novel *Legionella* species.

Physiological, biochemical, and morphological features

All three isolates were negative for Gram and Ziehl–Neelsen staining. Light microscopy confirmed motility. Biochemical tests revealed positive results for oxidase and gelatinase, while hippurate hydrolisis and catalase activity were negative. No β-lactamase production was detected. Table 3 summarizes the main biochemical features of strains 31fI33^T, 29fVS95, and 28fT52, compared with closely related *Legionella* species and *Lp*1 (Thacker et al., 1989; Brenner et al., 1985; Edelstein et al., 1982; Gorman et al., 1985; Journal et al., 1980). Table 4 presents a comparative overview of biochemical tests for strains 31fI33^T, 29fVS95, 28fT52, *L. feeleii* (ATCC 35072^T) and *Lp*1 (ATCC 33152^T). CFAs analysis revealed a dominant presence of Summed Features 3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c, 28.9%), followed by methyl-branched fatty acids $C_{16:0}$ iso (18.4%) and $C_{15:0}$ anteiso (15.4%). The CFA profile of the strains closely resembled that of *L. feeleii* (DSM 25316^T), (Table S2). Similarly, isoprenoid quinones analysis showed ubiquinone Q13 as the predominant component (61.3%) (Table S3). Lipid analysis demonstrated that all three isolates, as well as *L. feeleii,* shared a major lipid profile comprising diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). Minor components, including unidentified aminophospholipids (APL), aminolipids (AL), and other lipids (L), were strain-dependent (Fig. S3). Putrescine was the only polyamine identified in the studied strains and in *L. feeleii*.

Sequencing gene and phylogenetic analyses

PCR-based sequencing of the 16S rRNA, *mip*, and *rpoB* gene showed 100% identity and coverage for 16S rRNA and *mip* across all three isolates. For the *rpoB* gene, coverage was 100% with sequence identities of 99% for both 29fVS95 and 28fT52.

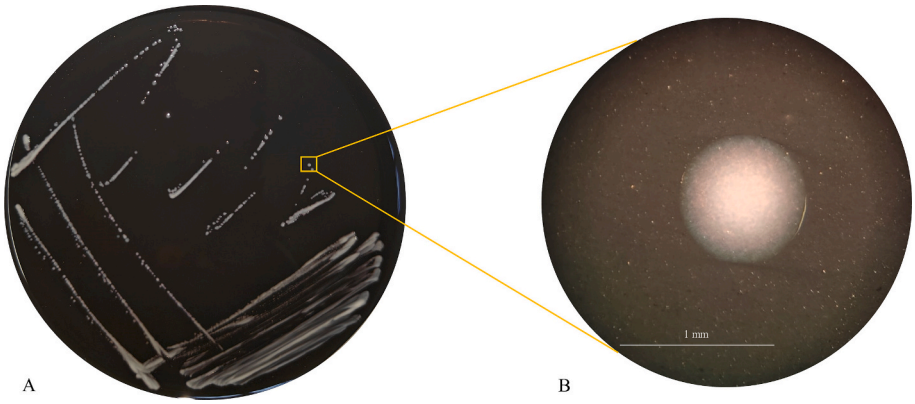

Fig. 1. Growth of strain 31fI33^T on BCYE Cys+ after 48 hours of incubation at 35 °C under 2.5% CO2 (A) and a representative single colony of strain 31fI33^T (B). Panel B was captured using a Heerbrugg Wild M38 Professional Optical Stereo Binocular Microscope with a Volpi Intralux 4000 Light Source (90W). The colony was observed at ×10 magnification, with a continuous zoom capability of ×4.5.

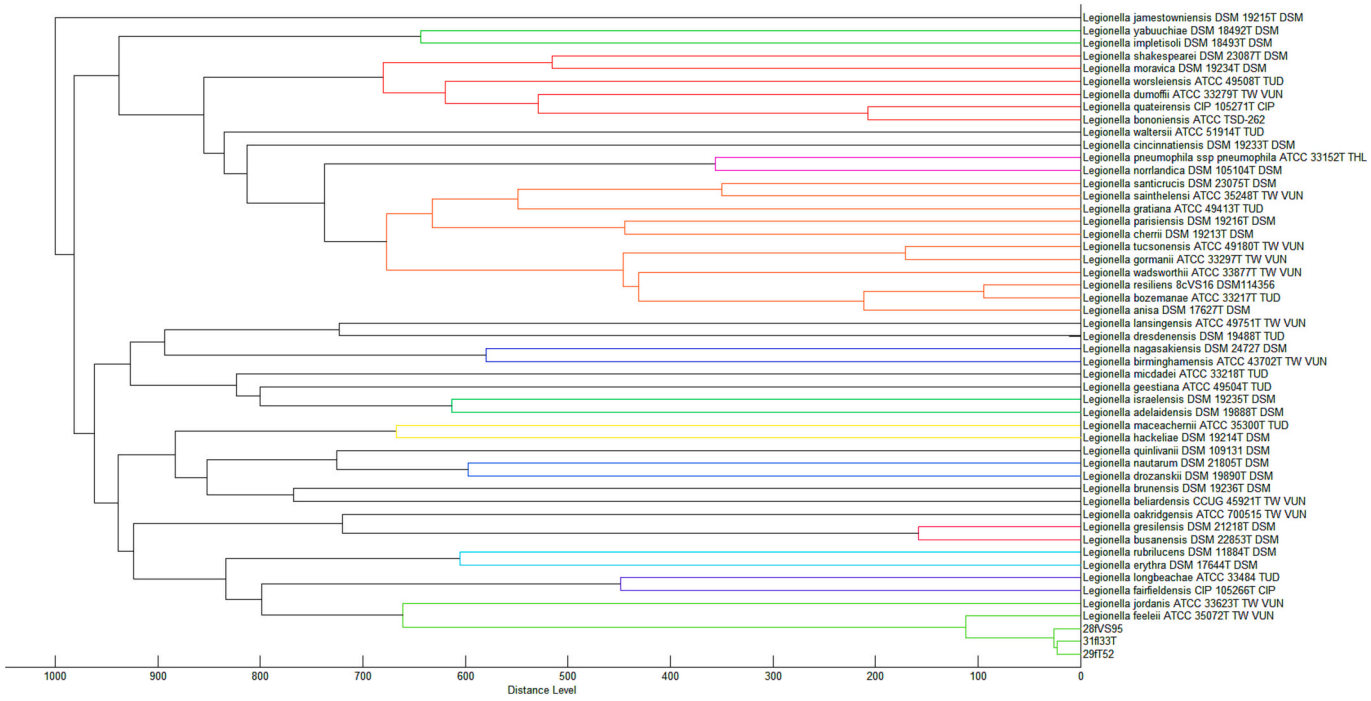

Fig. 2. Dendrogram based on whole-cell MALDI-TOF MS (MALDI Biotyper®, Bruker) comparing strains 31fI33^T, 29fVS95, and 28fT52 with the other *Legionella* strains present in instrument's database.

Table 5 lists the top ten matches for each gene sequences based on BLAST analysis against official *Legionella* species available in culture collections or in official database (Parte et al., 2020).

Sequence similarity for the 16S rRNA gene (1327 bp, WGS-derived) ranged from 96.23% to 99.08% with validated *Legionella* species, whereas *mip* (623 bp) and *rpoB* (329 bp) similarities ranged from 78.44% to 98.23% and from 80.67% to 95.00%, respectively. The highest similarity values were observed with *Legionella feeleii* ATCC 35072^T, with 98.23% identity for *mip* (11 DNA mismatches and one amino acid mismatch) and 95.00% identity for *rpoB* (16 DNA mismatches and no amino acid mismatches). According to the established species-level identification threshold (98.7% for 16S rRNA, 98.0% for *mip* and 95.2% for *rpoB*), the 16S rRNA similarity supports genus-level affiliation, whereas *mip* and *rpoB* values (98.23% and 95.00%, respectively) fall slightly below species-level cutoffs (98.0% and 95.2%, respectively), supporting the hypothesis that the isolates represent a novel species. Notably, although a 99.08% 16S rRNA similarity was observed with *L. tunisiensis* DSM 24805^T (NR_109416.1), this relationship was not supported by *mip* and *rpoB* analyses, confirming the higher discriminatory power of these markers for *Legionella* species identification (Ratcliff et al., 1998; Pascale et al., 2021).

Phylogenetic analysis based on the *mip*, *rpoB* and 16S rRNA genes sequences placed the three isolates in a distinct clade within the *L. feeleii* group (Figs. S4–S6).

Genome sequences were deposited in GenBank under the following accession numbers: 16S rRNA genes (OL441758.1, PV189399, and PV197233), *mip* genes (MW052974.1, PV223433.1, and PV223434.1), and *rpoB* genes (MZ367164, PV223437.1, and PV223438.1).

Whole genome sequencing and comparative analysis

The genome assembly and annotation data are summarized in Table 6. Briefly, the total genome size of strains 31fI33^T, 29fVS95, and 28fT52 was 3,281,701 bp, 3,627,471 bp, and 3,566,703 bp, respectively. Their GC contents were 41.50 mol%, 41.21 mol%, 41.24 mol%,

Table 3
Summary of the main biochemical characteristics of strains 31fI33^T, 29fVS95, 28fT52, compared with the most closely related *Legionella* species and *Lp*1 (+: positive, −: negative, +/-: Weakly or not always positive, Na: Not available).

Species	31fI33^T, 29fVS95, 28fT52	*L. feeleii*	*L. fairfieldensis*	*L. massiliensis*	*L. maceachernii*	*L. micdadei*	*L. drozanskii*	*L. nautarum*	*L. tunisiensis*	*Lp* 1
Accession number	**DSM 114357**	**ATCC 35072**	**ATCC 49588**	**DSM 24804**	**ATCC 35300**	**ATCC 33218**	**ATCC 700990**	**ATCC 49506**	**DSM 24805^T**	**ATCC 33152^T**
Catalase	−	+	+	+	+	+	+	+	+	+
Urease	−	−	−	**Na**	−	−	**Na**	−	−	−
Hippurate hydrolysis	−	+/−	−	+	−	−	**Na**	−		+
Oxidase	+	−	+	−	−	+	−	+	−	+
β-Lactamase production	−	−	−	**Na**	−	−	**Na**	+		+
Gelatin liquefaction (gelatinase)	+	−	−	−	+	−	**Na**	−	−	+
Glucose fermentation	−	−	−	−	−	−	−	−		−

Table 4
List of main biochemical features of 31fI33^T, 29fVS95, 28fT52 isolates, *L. feeleii* (ATCC35072^T) and *Lp*1 (ATCC 33152^T) (+: positive; -: negative).

Substrates	31fI33^T, 29fVS95, 28fT52	*L. feeleii*	*Lp*1	Substrates	31fI33^T, 29fVS95, 28fT52	*L. feeleii*	*Lp*1
ARA (arabinose)	−			**GLU (glucose)**	−	−	−
MNS (mannose)				**PRO (proline-β-naphthylamide)**	+	+	−
SUC (sucrose)				**PYR (pyrrolidine-b-naphthylamide)**	−	−	−
MEL (melibiose)				**GGT (g-Glutamyl b-naphthylamide)**	+	+	−
RHA (rhamnose)				**TRY (tryptophane b-naphthylamide)**	−	−	+
SOR (sorbitol)				**IND (tryptophane)**	−	−	−
MNT (mannitol)				**NO_3 (sodium nitrate)**	+	+	+
ADO (adonitol)				**GLR (p-nitrophenly β-glucuronide)**	−	−	−
ONPG (ρ-Nitrophenyl, b,D-galactoside)				**NAG (ρ-Nitrophenyl-N-acetyl-β,D-glucosaminide)**	−	−	−
PHO (p-nitrophenyl phosphate)	+	+	+	**GGL (γ-L-glutamyl p-nitroanilide)**	+	+	−
BGL (p-nitrophenyl α-β-glucoside)				**ESC (esculin)**	−	−	−
NPG (p-nitrophenyl β-galactoside)				**PHE (p-nitro-DL-phenylalanine)**	−	−	−
BPH (p-nitrophenyl bis-phosphate)				**URE (urea)**	−	−	+
BXY (p-nitrophenyl xyloside)				**CIT (citrate)**	−	−	−
AAR (p-nitrophenyl α-arabinoside)				**MLO (malonate)**	−	−	−
PHC (p-nitrophenyl phosphorylcholine)				**TTC (tetrazolium)**	−	−	−
ADH/ARG (arginine)				**LYS (lysine)**	−	−	−
TRD (aliphatic thiol)	+	+	+	**GLY (glycine)**	+	+	+
PHS (ρ-Nitrophenyl-phosphoester)	+	+	+	**BANA (N-Benzyl-arginine-b-naphthylamide)**	−	−	−
αGLU (ρ-Nitrophenyl-α,D-glucoside)				**EST (triglyceride)**	+	+	+
βGLU (ρ-Nitrophenyl-β,D-glucoside)				**INO (inositol)**	−	−	−
GAL (galactose)							

respectively. Genome completeness and contamination were within the accepted thresholds for high-quality genomes (≥90% completeness and ≤5% contamination), as proposed by Parks et al. (Parks et al., 2015). Specifically, strain 31fI33^T exhibited 99.84% completeness and 0.01% contamination; strain 29fVS95 exhibited 99.97% completeness and 1.76% contamination; and strain 28fT52 exhibited 99.98% completeness and 0.20% contamination. The corresponding GenBank accession numbers for the WGS data are JAJHHJ000000000 (31fI33^T), JBLZIM000000000 (29fVS95), and JBLZIL000000000 (28fT52), and the associated raw sequencing reads are publicly available through the associated BioProject and BioSample records in the NCBI Sequence Read Archive (SRA).

The percentage of conserved proteins (POCP), calculated across pairwise comparisons with closely related species, was approximately 60%, with mean values of 60.44%, 59.98% and 60.17% for strains 31fI33^T, 29fVS95, and 28fT52, respectively. These values support their placement within the genus *Legionella* and provide an initial genome-based framework for taxonomic assessment. While guidance thresholds have been proposed for genus delineation using AAI (e.g., >60–65%) and POCP (e.g., >50%), genus boundaries are known to be genus- and family-dependent and should not be interpreted as strict universal cut-offs without support from a coherent genome-based phylogeny (Riesco and Trujillo, 2024). In this regard, Riesco and Trujillo reported internal relatedness values within the family *Legionellaceae* (AAI ~68.8% and POCP ~59.3%), highlighting that family-level distributions can be informative but remain variable across taxa (Riesco and Trujillo, 2024). Accordingly, in the present study POCP and AAI were interpreted as complementary metrics supporting genus assignment, together with phylogenomic reconstruction, with detailed AAI results presented in Section 3.4.

Consistent with POCP results, whole-genome sequence (WGS)-based comparative analyses corroborated the findings obtained from gene-specific approaches. When compared with *L. feeleii* (ATCC 35072^T), the three strains showed sequence identities of 93.80% (31fI33^T),

Table 5
Top ten sequence similarities among 31fI33^T, 29fVS95, 28fT52 and other officially recognized *Legionella* type strains based on partial 16S rRNA, *mip* and *rpoB* gene comparisons. The gene fragment lengths used for comparison and the corresponding accession numbers are provided in parentheses.

Partial *16S* rRNA gene (1327 bp) (OL441758.2)	Identity percentage (%)	Coverage (%)	Partial *mip* gene (623 bp) (MW052974.1)	Identity percentage (%)	Coverage (%)	*rpoB* gene (329 bp) (MZ367164)	Identity percentage (%)	Coverage (%)
L. tunisiensis DSM 24805^T (NR_109416.1)	99.08%	98%	*L. feeleii* ATCC 35072^T (U92205.1)	98.23%	100%	*L. feeleii* ATCC 35072 ^{T} (AF367731.1)	95.00%	91%
L. feeleii ATCC 35072^T (NR_026117)	98.12%	96%	*L. tunisiensis* DSM 24805^T (JN191172.1)	93.57%	100%	*L. tunisiensis* DSM 24805^T (LR134173.1)	86.93%	100%
L. massiliensis DSM 24804^T (NR_109415)	97.31%	98%	*L. clemsonensis* CDC D5610^T (KT369126)	83.89%	92%	*L. hackeliae* ATCC 35250^T (LN681225.1)	81.46%	100%
L. adelaidensis NCTC 12735^T (LR134413.1)	96.62%	100%	*L. cardiaca* ATCC BAA-2315^T (JF831048.1)	84.33%	83%	*L. jordanis* NCTC 11533^T (LR134383.1)	81.46%	100%
L. cardiaca ATCC BAA-2315^T (CP119078)	96.54%	100%	*L. brunensis* ATCC 43878^T (U92227.1)	81.58%	91%	*L. clemsonensis* CDC D5610^T (CP16397.1)	81.35%	99%
L. spiritensis NCTC 11990^T (LT906457.1)	98.80%	97%	*L. rowbothamensis* ATCC 700991^T (AF148984.1)	79.22%	86%	*L. lytica* CCUG 112^T (CP071527.1)	81.35%	99%
L. fallonii ATCC 700992^T (LN614827.1)	96.38%	100%	*L. maceachernii* ATCC 35300^T (U92211.1)	79.21%	85%	*L. oakgridgensis* ATCC 33761^T (CP004006.1)	81.29%	99%
L. sainthelensi NCTC 11988^T (LR134388.1)	96.31%	100%	*L. lytica* IMVS 3376^T (AF148982.1)	78.75%	87%	*L. maceachernii* ATCC 35300^T (AF367742.1)	83.45%	90%
L. dresdenensis DSM 19488^T (NR_115062.1)	97.06%	97%	*L. drancourtii* ATCC 50991^T (AF487978.1)	78.10%	89%	*L. cardiaca* ATCC BAA-2315^T (CP119078.1)	80.73%	99%
L. oakridgensis NTCT 11531^T (LR134286.1)	96.23%	100%	*L. bozemanii* ATCC 33217^T (U91609.1)	78.44%	86%	*L. waltersii* NCTC 13017^T (LT906442.1)	80.67%	99%

Table 6
Genome statistics data from annotation for 31fI33^T, 29fVS95, and 28fT52

Attribute	Data for strain 31fI33^T	Data for strain 29fVS95	Data for strain 28fT52
No. of raw reads	763,324	772,696	874,914
Avg read length (bp)	243	191	250
Coverage (×)	99.0	85.0	125
Total Length (bp)	3,281,701	3,627,471	3,566,703
No. of contigs	159	489	161
GC Content (mol%)	41.5	41.21	41.24
N_{50} (bp)	153,130	46,211	116,682
L_{50} (bp)	7	25	10
No. of coding sequences	2968	3234	3270
No. of rRNAs (complete and partial)	1	23	11
No. of tRNAs	41	44	42

93.89% (29fVS95), and 93.81% (28fT52), while ANIm values were 94.06%, 94.05%, and 94.06%, respectively. dDDH, calculated using formula 2, yielded values of 54.90% for strain 31fI33^T, 55.00% for strain 29fVS95, and 54.90% for strain 28fT52.

These values fall below the accepted thresholds for species delineation (95% for ANI and 70% for dDDH), supporting the hypothesis that strains 31fI33^T, 29fVS95, and 28fT52 represent a novel *Legionella* species distinct from *L. feeleii* (ATCC 35072^T) (Stackebrandt and Goebel, 1994; Stackebrandt and Ebers, 2006). Phylogenetic trees based on WGS data revealed a monophyletic group that includes the studied isolates together with *L. feeleii* and *L. tunisiensis* (Fig. 3). However, within this group, the isolates formed a clearly distinct clade, further supporting their classification as a distinct species.

In agreement with these results, genome-based phylogenomic analysis performed using Type (strain) Genome Server (TYGS), which implements the GBDP approach, supports the placement of strains 31fI33^T, 29fVS95 and 28fT52 as a distinct and coherent lineage within the genus *Legionella*, consistent with ANI- and dDDH-based species delineation. Consistently, GTDB-based phylogenomic analysis placed our strains within the *Legionella/Tatlockia* clade, clustering in the same lineage as *Tatlockia feeleii* and *Tatlockia tunisiensis*. Due to the size and complexity of the GTDB-Tk phylogenomic output, the complete tree is provided as Supplementary Data (Supplementary File–GTDB-Tk output tree (iTOL-compatible)) in GTDB-Tk/iTOL-compatible format and therefore cannot be displayed as a static figure. The file can be explored using the GTDB-Tk toolkit or interactive tree visualization software, while the placement of the study strains within the relevant clade is described in the Results section.

ANIm comparisons among our isolates showed that strains 29fVS95 and 28fT52 shared 99.34% and 99.87% similarity with strain 31fI33^T, respectively. The similarity between 29fVS95 and 28fT52 was 99.33%. These high values confirm that the three strains belong to the same species and are highly similar at the genomic level.

Clonality assessment experiments using RAPD-PCR and GTG-PCR indicated that the three isolates are not clonal (Fig. S7). However, whole-genome SNP analysis revealed substantial genetic differences among them, as follows: 8,717 SNPs between 29fVS95 and 31fI33^T, 1,688 SNPs between 28fT52 and 31fI33^T, and 9,281 SNPs between 29fVS95 and 28fT52. Given that thresholds to indicate a recent transmission or clonality are typically below 25 SNPs, these findings suggest that the isolates are genetically distinct and not clonal (Fang et al., 2021).

Core genome analysis

The core genomic features of the three isolates, compared with the most closely related *Legionella* species are presented in Table 7. Genomic information for the comparative strains was retrieved from NCBI.

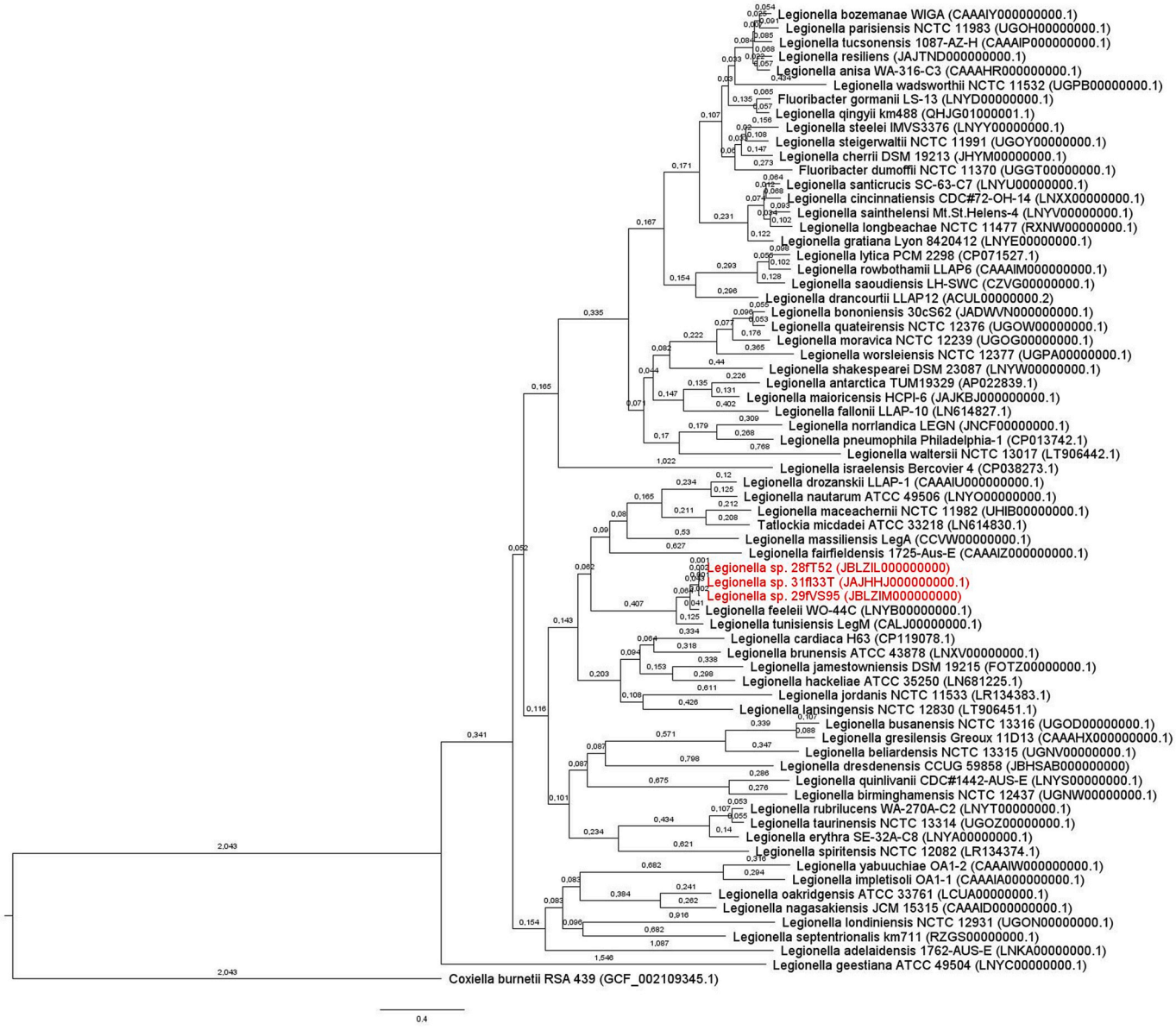

Fig. 3. WGS-based phylogenetic relationships among strains 31fl33^T, 29fVS95, 28fT52 and 65 validated *Legionella* species genome annotated in NCBI. The maximum-likelihood tree was inferred with RaxML (v8.2.11) with rapid bootstrapping (100 replicates) and rooted using Coxiella burnetii RSA 493 as the outgroup. Branch lengths represent substitutions per site (scale bar: 0.2 substitutions per nucleotide position). Our strains are shown in red; bootstrap support values are displayed at the nodes.

Table 7
Synopsis of the basic genomic data of 31fl33^T, 29fVS95, 28fT52, the most related *Legionella* species (main clade) and *Lp*1.

Type Strains	Taxon name	GenBank accession ID	No. of contigs	Size (Mbp)	GC (mol%)	No. of CDS	No. of rRNA	No. of tRNA
DSM 114357^T	31fl33^T	JAJHHJ000000000.1	3	3.2	41.5	2968	1	41
/	29fVS95	JBLZIM000000000.1	489	3.6	41.21	3234	20	44
/	28fT52	JBLZIL000000000.1	161	3.5	41.24	3270	6	42
DMS 24588^T	*L. tunisiensis*	CALJ00000000.1	340	3.4	40	5.146	3	36
ATCC 35072^T	*L. feelei*	CAAAHT000000000.1	150	3.5	41	3.532	2	42
ATCC 49588^T	*L. fairfieldensis*	CAAAIZ000000000.1	76	2.7	39.6	2.656	2	42
ATCC 3218^T	*L. micdadei*	FMVN00000000.1	27	3.3	40.4	3.085	4	36
NCTC 11982^T	*L. maceachernii*	UHIB00000000.1	4	3.7	40.5	3.482	3	45
ATCC 49506^T	*L. nautarum*	LNYO00000000.1	27	3.6	39.6	3.345	2	33
NCTC 14628^T	*L. drozanskii*	CAAAIU000000000.1	64	3.6	39.3	3.360	2	41
DMS 24804^T	*L. massiliensis*	CCVW00000000.1	8	4.4	41.1	3.860	7	40
ATCC 33152^T	*Lp1*	GCA_000008485.1	Complete genome	3.4	38.5	2.995	9	44

Comparative genomic analysis was performed using BRIG (Alikhan et al., 2011), with strain 31fl33^T as the reference and including 28fT52,

29fVS95, *L. feeleii* (the most closely related species), and *L. pneumophila* (the more pathogenic species). BRIG analysis revealed regions of complete identity with the reference genome (dark shading, 100%), as well as region with moderate similarity (medium shading, 70%) and lower similarity (light shading, 50%). White areas correspond to regions with less than 50% sequence identity or absent sequences. These variable regions appear as discontinuities or lighter areas across the concentric rings representing the different genomes (Figure S8). Using an in-house Phyton script applied to the BRIG output, genome coverage percentages were calculated. Strains 28fT52 and 29fVS95 showed extensive homology with strain 31fI33^T, with coverage values of 98.84% and 97.37%, respectively, and only limited regions lacking coverage. In contrast, a higher number of gaps was detective in the genome of *L. feeleii* (coverage of 94.71 %) and *L. pneumophila* (coverage of 44.66%), reflecting the presence of strain-specific regions absent from the reference genome. Overall, these findings indicated a conserved core genome among the closely related isolates, while highlighting the increasing genomic variability associated with the more distantly related *Legionella* species (Svetlicic et al., 2023). Pangenome analysis placed strains 31fI33^T, 29fVS95, and 28fT52 in a distinct clade, separate from other *Legionella* species (Figure 4), indicating evolutionary differentiation. Gene distribution analysis revealed substantial genomic variability, with some gene sets shared among our strains and others restricted to specific subclades. Among strains 31fI33^T, 29fVS95, and 28fT52, there is a share of 3,570 total genes (present in all isolates), divided into 2,788 core genes and 782 shell genes. Genomic diversity increased progressively with the phylogenetic distances, resulting in fewer shared genes among more distantly related species. When considering only the *Legionella* species most closely related to our strains, together with *L. pneumophila,* the most studied and virulent species, the dataset comprised 29,066 total genes, including 12 core genes, 4,045 shell genes, and 25,009 cloud genes. These data underscore the high genomic diversity within the genus *Legionella*, as also demonstrated by the AAI analysis (Figure 5) and BRIG analysis (Figure S8). The AAI profile of our strains within the clade (*L. feeleii*, *L. tunisiensis,* and our strains) was fully consistent with both the pangenome analysis and the phylogenetic trees. AAI values for all 65 *Legionella* species are reported in Table S4, with values corresponding to our strains and closely related species highlighted in bold red. The complete list of species, along with their Assembly and GenBank accession IDs, is provided in Table S1. The AAI heatmap (Figure 5) illustrates genome similarity using a color gradient ranging from white (highest similarity) to dark red (lowest similarity). AAI values among strains 31fI33^T, 29fVS95, 28fT52 and *L. feeleii* WO-44C DSM25316^T ranged from 95.23% to 95.37%, whereas lower values were observed with *L. tunisiensis* LegM DSM24805^T (89.83–90.08%). In contrast, AAI values among our strains ranged from 99.35% to 99.87%, exceeding the established interspecies threshold of approximately 95–96% and clearly indicating that these strains belong to the same species (Konstantinidis and Tiedje, 2005).

Virulence, pathogenicity and antibiotics resistance gene results

Table S4 summarizes the distribution of virulence-associated genes in strains 31fI33^T, 29fVS95, and 28fT52, as well as reference strains from related *Legionella* clades, including *L. feeleii* WO-44C DSM25316^T, *L. maceachernii* PX-1-G2-E2 NCTC11982^T, *L. micdadei* TATLOCK ATCC33218^T, *L. nautarum* 1224 ATCC49506^T, *L. drozanskii* LLAP-1 ATCC700990^T, *L. fairfieldensis* 1725-Aus-E ATCC49588^T, *L. massiliensis* LegA DSM 24804^T, *L. tunisiensis* LegM DSM 24805^T. Additional comparison included *Legionella* species listed in the VFDB database: *L. longbeachae* NSW150 (NC_013861), *L. pneumophila* str. Corby (NC_009494), *L. pneumophila* str. Lens (NC_006369), *L. pneumophila* str. Paris (NC_006368), *L. pneumophila subsp. pneumophila* str. Philadelphia 1 (NC_002942). Analysis presented in Table S4 showed that strains 31fI33^T, 29fVS95, and 28fT52 harbor a conserved suite of virulence factors, notably those related to key functional categories such as the *Dot/Icm* type IVB secretion system (e.g., *dotA, dotC, icmB/dotO, icmC/dotE, icmD/dotP, icmE/dotG*), iron acquisition systems (e.g., *feoA, feoB*), chaperone-usher pili systems (e.g., *pilB, pilC, pilD, pilQ*), and general stress response regulators (e.g., *csrA, rpoS, relA*). Despite this conserved virulence core, several genes associated with additional intracellular survival strategies were absent in all three strains, including effectors of the *sidE/laiD* family, components of the *lvh* type IVA secretion system, and genes from the *rml* operon involved in LPS biosynthesis.

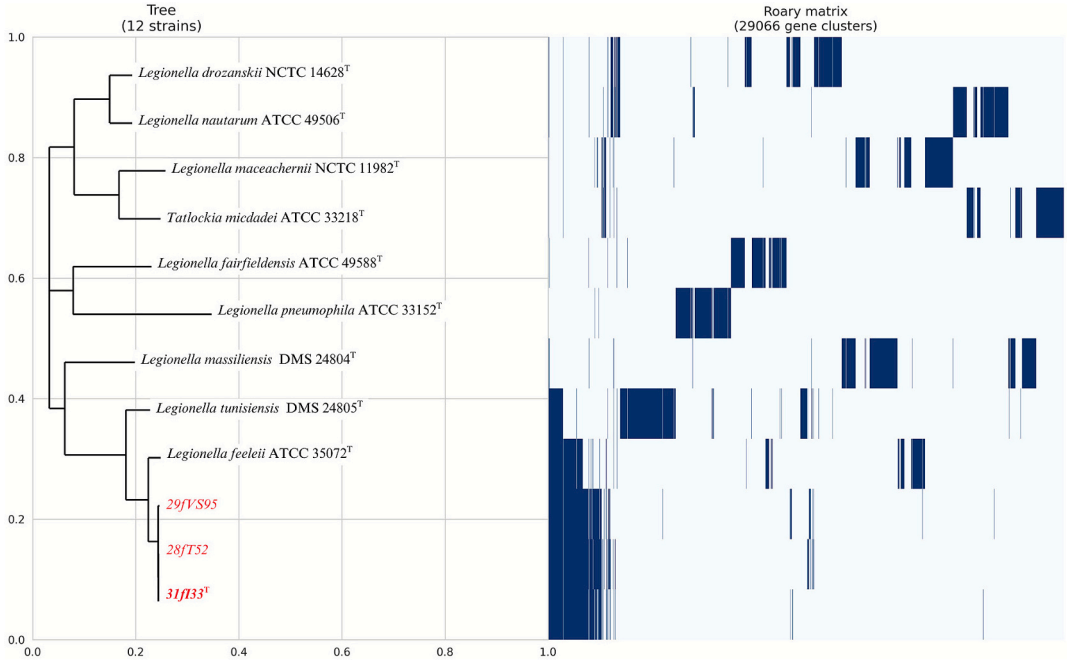

Fig. 4. Pangenome analysis of the strains 31fI33^T, 29fVS95, 28fT52, the close phylogenetic relative strains (*L. tunisiensis*, *L. feeleii*, *L. fairfieldensis, L. micdadei, L. maceachernii, L. nautarum, L. drozanskii* and *L. massiliensis*), and *Lp1,* performed using Roary software. WGS were clustered based on the presence or absence of core genes. Blue indicates gene presence, white the absence.

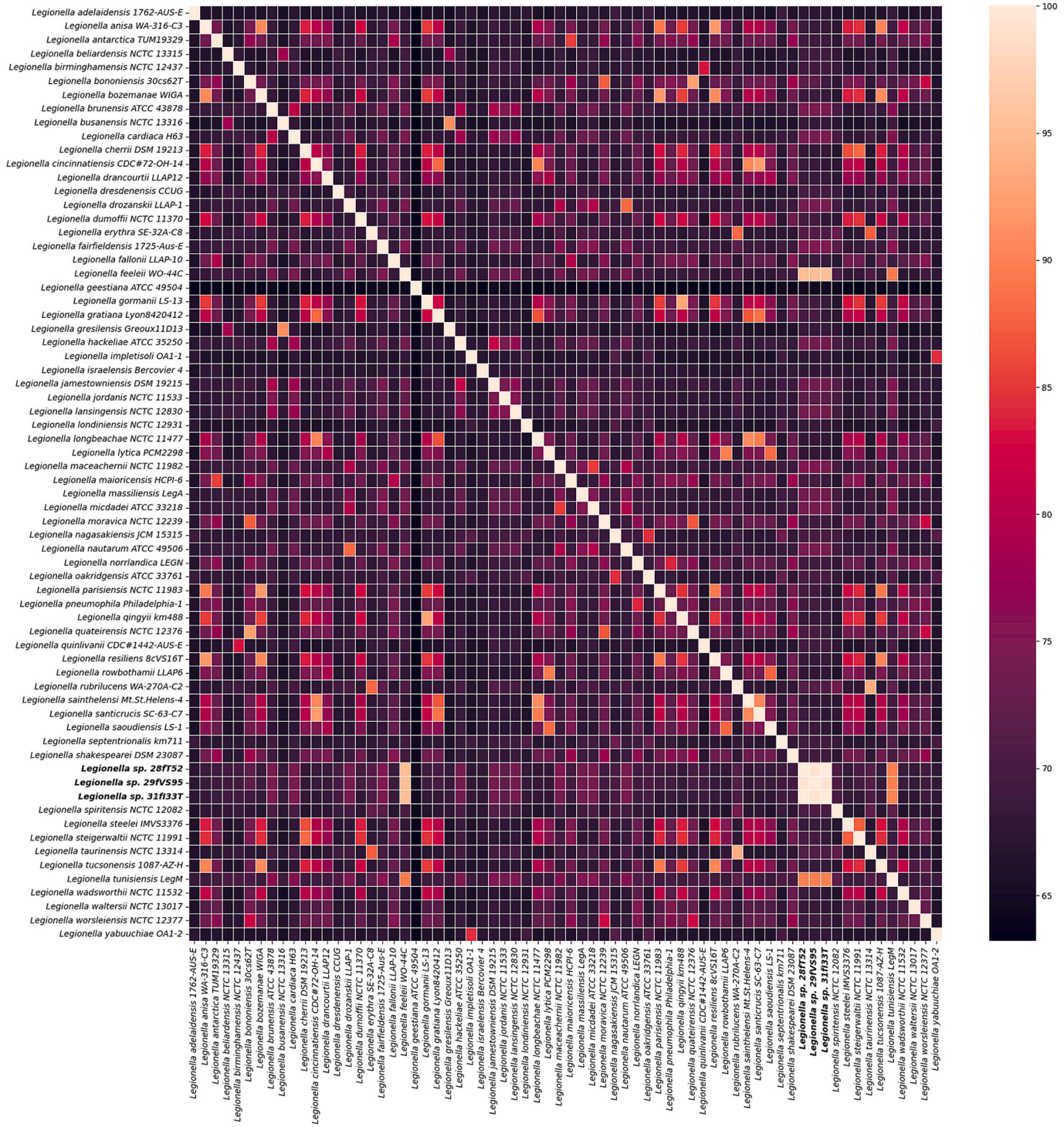

Fig. 5. Amino acid composition heatmap (AAI data) of 31fI33^{T}, 29fVS95, and 28fT52 strains and the other 65 *Legionella* species genome presented in NCBI.

Interestingly, strain-specific findings included *acrB*, a multidrug efflux gene, found only in 29fVS95. The *acrB* gene plays a key role in quinolones resistance and may also enhance persistence within macrophages (Padilla et al., 2010; Fanelli et al., 2023). In contrast, the *orfN* gene was uniquely detected in 28fT52. While its function in *Legionella* is uncharacterized, in *P. aeruginosa orfN* contributes to O-antigen biosynthesis (King et al., 2009), suggesting a potential, but yet unconfirmed, role in LPS modification and immune evasion in *Legionella*. A conserved core of *Dot/Icm* secretion system genes and stress-related regulators was detected across all analyzed species, emphasizing their central role in *Legionella* pathogenicity. PathogenFinder analysis supported the pathogenetic potential of these strains, with predicted probabilities of human pathogenicity of 82.4% (31fI33^{T}), 78.8% (29fVS95), and 77.2% (28fT52).

Amplicon-based 16S rRNA gene metabarcoding community profiling results

Taxonomic profiling of the bacterial community was obtained through the V3-V4 region of the 16S rRNA gene amplicon sequencing of

the water samples. This approach provided taxonomic resolution and detected a total of 168 bacterial genera, several of which are clinically significant. Specifically, taxa with a low relative abundance (<1.0%) were grouped as "Others" in Figure 6. Among the detected taxa, clinically relevant genera included *Mycobacterium* (18.34%), *Legionella* (1.14%), and *Stenotrophomonas* (0.54%), known for their roles in respiratory and opportunistic infections, as well as zoonotic genera such as *Leptospira* (0.27 %) and *Coxiella* (0.10%). Opportunistic pathogens with emerging clinical relevance in nosocomial setting, including *Delftia* (2.20%), *Brevundimonas* (0.24%), and *Roseomonas* (0.08%) were also detected (Falkinham et al., 2015; Ryan and Pembroke, 2018; Wang et al., 2012; Doughari et al., 2011). Within the *Legionella* genus, the detected species included *L. pneumophila* (0.67%), *L. birminghamensis* (0.31%), *L. massiliensis* (0.07%), *L. qingyii* (0.05%), *L. shakespearei* (0.02%), *L. maceachernii* (0.01%), and *L. impletisoli* (0.01%).

Moreover, to further explore the potential ecological niches and environmental distribution of the strains 31fI33^T, 29fVS95, and 28fT52, the Protologger web tool was applied to their genomes (Hitch et al., 2021). Interestingly, the database search yielded no metagenome-assembled genomes (MAGs) with sequences matching any of the three strains under study.

This outcome is consistent with the intrinsic limitations of 16S rRNA gene–based metabarcoding approaches for resolving closely related *Legionella* species, due to their high sequence homology. Accordingly, species-level discrimination within the genus *Legionella* is not possible using 16S rRNA gene sequences alone, due to the high level of sequence homology among closely related species, in accordance with Pascale et al. (Pascale et al., 2021). However, no matches were detected even when WGS sequences were used as queries, suggesting that these strains may be absent from currently available public metagenomic datasets. These findings highlight the importance of further studies to better understand *Legionella* diversity and host-microbe dynamics among ecologically distinct strains. In addition, Protologger analysis revealed the presence of 126 CAZymes, including several glycoside hydrolase families, supporting the predicted utilization of both starch and glucose as carbon sources.

Antibiotic susceptibility test results

The MICs for strains 31fI33^T, 29fVS95, and 28fT52 and the reference strain *Lp*1 (ATCC 33152^T) were determined using gradient diffusion and microbroth dilution methods. The results are presented in Tables 8 and 9. Notably, the strains demonstrated reduced sensitivity to azithromycin, with MIC value of 0.5 mg/L and 1 mg/L, as determined by gradient diffusion and microbroth dilution methods, respectively. This diminished susceptibility may be linked to the potential involvement of efflux pump mechanisms, highlighting the need for further investigation into their role in the observed phenotype (Cocuzza et al., 2021).

Ecological and epidemiological considerations

The detection of a novel *Legionella* species in our region has garnered

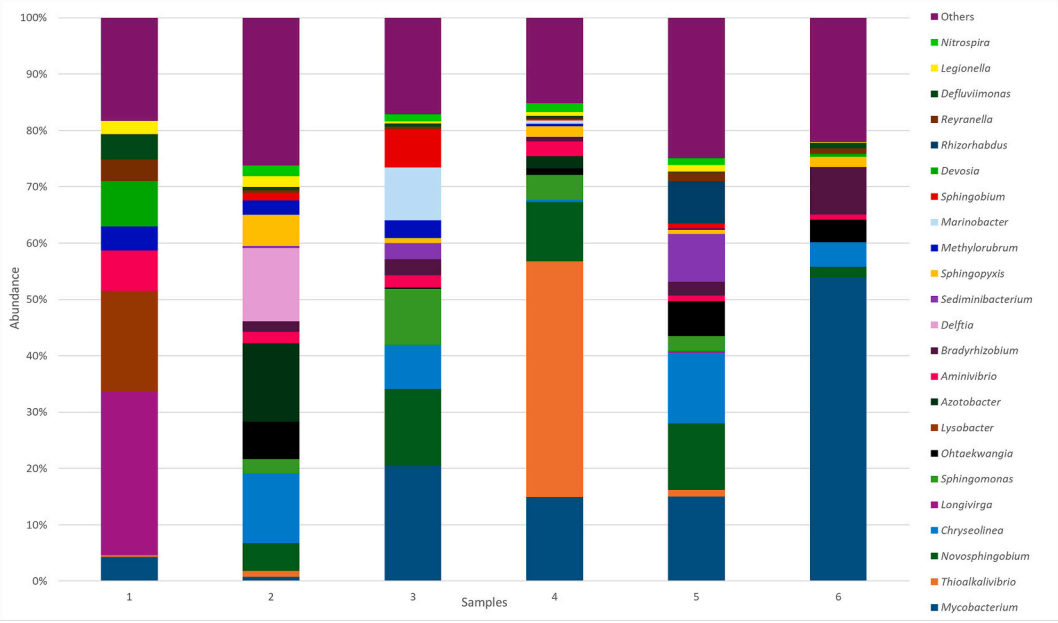

Fig. 6. Relative abundance profile (%) of bacterial community (at the genus level), detected in water samples from the three facilities through 16S rRNA gene metagenomic analysis. Samples 1-6 represent two representative outlets for each building (samples 1 and 2 from facility A, samples 3 and 4 from facility B, and samples 5 and 6 from facility C).

Table 8
MICs of 31fI33^T, 29fVS95, and 28fT52 strains and *Lp1* to antimicrobial drugs (gradient MIC method).

Antimicrobial	Concentration range tested (mg/L)	MIC for 31fI33^T, 29fVS95, and 28fT52	Interpretation	MIC for *Lp*1 ATCC 33152^T	Interpretation	*Lp* EUCAST Cut-off (not standardized)
Azithromycin	0.016-256	0.5	R	0.125	S	0.25
Ciprofloxacin	0.002-32	0.38	S	0.75	S	0.5
Doxicicline	0.016-256	0.75	S	N.D.	/	8
Erythromycin	0.016-256	0.25	S	0.125	S	0.5
Levofloxacine	0.002-32	0.25	S	N.D.	/	0.25
Rifampicin	0.002-32	0.016	S	0.023	S	0.032

N.D.: not detected; R: resistant; S: sensible according to EUCAST guidance document.

Table 9
MICs of 31fl33^T, 29fVS95, and 28fT52 strains and *Lp*1 to antimicrobial drugs (microbroth dilution method).

Antimicrobial	Concentration range tested (mg/L)	MIC for our strains	Interpretation	MIC for *Lp1* ATCC 33152^T	Interpretation	*Lp* EUCAST Cut-off (not standardized)
Azithromycin	0.0075-16	1	R	0.5	R	0.125
Erythromycin	0.0075-16	0.5	S	1	S	1
Ciprofloxacin	0.00025-0.512	0.032	S	0.032	S	0.032

R: resistant; S: sensible according to EUCAST guidance document.

considerable interest and warrants further investigation, particularly as this *Legionella* species represents the third newly described *Legionella* species identified in Italy, specifically in the area surrounding the city of Bologna. The surveillance strategies outlined by European, National and Regional Directives have led to an increase in environmental surveillance also as self-assessment especially for companies and health-care facilities. This improvement in monitoring efforts not only improves control over *Legionella* prevalence within facilities but also strengthens epidemiological investigations during outbreaks and clusters. Regarding the epidemiology of Legionellosis in Europe and Italy, *L. pneumophila* remains the principal species of concern, being isolates in 90% of cases in Europe and 100% in Italy (Rota et al., 2023; European Centre for Disease Prevention and Control (ECDC), 2023). Non-*pneumophila* *Legionella* species account for approximately 3% of cases, most commonly associated with *L. anisa*, *L. bozemanii*, *L. longbeachae*, *L. micdadei*, and *L. cincinnatiensis*. An additional 14 clinical isolates have

Table 10
Description of *Legionella petroniana* sp. nov.

Guiding Code for Nomenclature	ICNP
Nature of the type material	**31fl33^T:** JAJHHJ000000000 **29fVS95:** JBLZIM000000000 **28fT52:** JBLZIL000000000
Genus name	*Legionella*
Species name	*Legionella petroniana*
Genus status	–
Genus etymology	–
Type species of the genus	–
Specific epithet	petroniana
Species status	sp. nov.
Species etymology	pe.tro.ni.a'na. N.L. fem. adj. petroniana, pertaining to Saint Petronio, the protector of Bologna city
Designation of the Type Strain	31fl33^T
Strain Collection Numbers	= DSM 114357^T=CCUG 76442^T
Designated Genome, MAG or SAG	31fl33^T, 29fVS95 and 28fT52
Type Genome, MAG or SAG accession Nr. [INSDC databases]	**31fl33^T:** JAJHHJ000000000 **29fVS95:** JBLZIM000000000 **28fT52:** JBLZIL000000000
Access to raw data (e.g. SRA accession)	**31fl33^T:** SRX12762786 **29fVS95:** SRX27864002 **28fT52:** SRX27864003
Registry number	-
Genome status	Incomplete
Genome size	**31fl33^T:** 3,282 kbp **29fVS95:** 3,627 kbp **28fT52:** 3,567 kbp
GC mol%	**31fl33^T:** 41.50 **29fVS95:** 41.21 **28fT52:** 41.24
16S rRNA gene accession nr.	**31fl33^T:** OL441758.1 **29fVS95:** PV189399 **28fT52:** PV197233
Description of the new taxon and diagnostic traits	Cells are Gram-stain-negative, Ziehl Neelsen-negative, rod-shaped, and motile. The temperature range for growth is 32–37 °C, with no significant differences observed across this range. Cells are aerobic and are able to grow on BCYE Cys+, GVPC and MWY agar, but no growth was observed on non-selective enriched media. The species is positive for oxidase and gelatinase and shows no β-lactamase activity. The predominant fatty acids are Summed features 3 (C16:1ω7c/C16:1ω6c), C16:0 iso and C15:0 anteiso. Q13 is the major ubiquinone. Major lipids included diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). Putrescine is the only polyamine detected. The species was isolated from both cold and hot WDS in one company and two hospitals located in Bologna, Italy.
Country of origin	Italy
Region of origin	Emilia-Romagna
Date of isolation (dd/mm/yyyy)	**31fl33^T:** October 2018 **29fVS95:** July 2019 **28fT52:** April 2021
Source of isolation	Hot and cold-water distribution systems
Sampling date (dd/mm/yyyy)	**31fl33^T:** October 2018 **29fVS95:** July 2019 **28fT52:** April 2021
Latitude (xx°xx′xx″N/S)	–
Longitude (xx°xx′xx″E/W)	–
Altitude (meters above sea level)	–
Number of strains in study	–
Source of isolation of non-type strains	–
Information related to the Nagoya Protocol	Not applicable

been identified only at the genus level, as *Legionella* species unknown. Since 2020, according to the scientific literature, the last five novel *Legionella* species have been reported by Italian, Spanish, Japanese, and Chinese research groups (Crespi et al., 2023; Girolamini et al., 2022c; Li et al., 2021; Cristino et al., 2024). This likely reflects a growing research focus on species-level identification and genomic characterization, rather than solely on bacterial quantification. The increase in the number of *Legionella* species reported is undoubtedly linked to the widespread use of next-generation sequencing technologies and advanced bioinformatics tools, which facilitate rapid genome comparison, species delineation, and global tracking of isolates.

Although it is not the main focus of this study, the experience developed in the *Legionella* surveillance and characterization, emphasize the need to improve the standardized culture-based techniques to a careful observation of colonies, especially when morphological traits are atypical. In recent years, several *Legionella*-like isolates have been described with unusual phenotypes: very small colonies, with color from white to gray, and often with atypical fluorescence patterns. These atypical colonies frequently do not react with available commercial antisera and can only be correctly identified through subculture on BCYE Cys+/- media. A recent study published by Gabrielli et al. (Gabrielli et al., 2025) has highlighted significant genomic variability within the genus, underscoring both the limited current knowledge and the potential health implications of lesser-known *Legionella* species. The notable diversity between environmental and clinical strains challenges traditional surveillance paradigms and suggests that greater attention should be directed toward identifying atypical isolates from specific environments.

In the context of climate change, our findings highlight the importance of considering environmental factors in the ecology of novel *Legionella* species. As global temperatures continue to rise, particularly in Mediterranean regions, ongoing surveillance and genomic monitoring become essential to understanding how such conditions may influence the emergence, persistence, and pathogenic potential of both known and previously unrecognized species. In the recent years the number of *L-np* species isolated from the environment has been increasing, and the presence in the Emilia-Romagna region of Po Valley, and Po River along with recent heavy rain and floods events, probably contributes to creating an ecological environment (temperature, relative humidity and vapor pressure) able to support *Legionella* growth and differentiation, according to several studies (Beauté et al., 2016; Fischer et al., 2023; Gleason et al., 2016; Braeye et al., 2020). In line with Wade et al., peaks of Legionnaires' Disease in Italy are reported in late summer and early fall, suggesting that weather conditions may affect transmission. Studies conducted in several other countries, such as Belgium, the Netherlands, the United Kingdom, Taiwan, Spain, Switzerland, New Zealand, Korea, Japan, the United States, and several European countries confirmed this hypothesis (Wade and Herbert, 2024). The data report that 3 cm of precipitation increased the odds of legionellosis more than four times.

Considering the extreme weather conditions, LD infections are likely to increase in future years. Therefore, the ecological mapping of these new species and attention to atypical *Legionella* isolates are fundamental to tracing epidemiological events and planning the best strategies to protect public health.

Conclusions

In conclusion, based on the genotypical and phenotypical evidences, strains 31fI33^T, 29fVS95, and 28fT52 represent a novel species belonging to the genus *Legionella*, for which we propose the name *Legionella petroniana* sp. nov. (Table 10).

The identification of this species expands the knowledge of diversity within the *Legionella* genus and provides new insights not only into its ecological distribution, but also into its potential virulence and antibiotic resistance, with potential implication in clinical contexts. The integration of phenotypic characterization with genome-based analyses enabled the delineation of this novel species and highlighted the limits of traditional approaches.

Overall, these findings highlight the importance of enhancing environmental surveillance by combining gold-standard culture methods with genomic tools to improve detection and enable more accurate classification and characterization of *Legionella* species.

All authors have read and agreed to the published version of the manuscript.

CRediT authorship contribution statement

Sandra Cristino: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Data curation, Conceptualization. **Laura Caligaris:** Writing – original draft, Validation, Formal analysis, Conceptualization. **Silvano Salaris:** Software, Methodology, Formal analysis. **Carlo Derelitto:** Software, Formal analysis. **Caterina Bonincontro:** Investigation, Formal analysis. **Federica Marino:** Investigation, Formal analysis. **Antonella Grottola:** Methodology, Investigation, Conceptualization. **Luna Girolamini:** Writing – original draft, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2026.126694.

Data availability

The GenBank accession numbers for the strains are the follows:

31fI33^T

- 16S rRNAs: OL441758.1
- *mip*: MW052974.1
- *rpoB*: MZ367164
- WGS: JAJHHJ000000000

29fVS95

- 16S rRNAs: PV189399
- *mip*: PV223433.1
- *rpoB*: PV223437.1
- WGS: JBLZIM000000000

28fT52

- 16S rRNAs: PV197233
- *mip*: PV223434.1
- *rpoB*: PV223438.1
- WGS: JBLZIL000000000

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